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Strain-specific impact of PsaR of *Streptococcus pneumoniae* on global gene expression and virulence

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Previous studies have indicated that PsaR of *Streptococcus pneumoniae* is a manganese-dependent regulator, negatively affecting the expression of at least seven genes. Here, we extended these observations by transcriptome and proteome analysis of *psaR* mutants in strains D39 and TIGR4. The microarray analysis identified three shared PsaR targets: the *psa* operon, *pcpA* and *prtA*. In addition, we found 31 genes to be regulated by PsaR in D39 only, most strikingly a cellobiose-specific phosphotransferase system (PTS) and a putative bacteriocin operon (*sp0142–sp0146*). In TIGR4, 14 PsaR gene targets were detected, with the *rlrA* pathogenicity islet being the most pronounced. Proteomics confirmed most of the shared gene targets. To examine the contribution of PsaR to pneumococcal virulence, we compared D39 and TIGR4 wild-type (wt) and *psaR* mutants in three murine infection models. During colonization, no clear effect was observed of the *psaR* mutation in either D39 or TIGR4. In the pneumonia model, small but significant differences were observed in the lungs of mice infected with either D39wt or Δ*psaR*: D39Δ*psaR* had an initial advantage in survival in the lungs. Conversely, TIGR4Δ*psaR*-infected mice had significantly lower bacterial loads at 24 h only. Finally, during experimental bacteraemia, D39Δ*psaR*-infected mice had significantly lower bacterial loads in the bloodstream than wt-infected mice for the first 24 h of infection. TIGR4Δ*psaR* showed attenuation at 36 h only. In conclusion, our results show that PsaR of D39 and TIGR4 has a strain-specific role in global gene expression and in the development of bacteraemia in mice.

INTRODUCTION

Streptococcus pneumoniae encounters different environments during its life cycle: in most cases it inhabits the human nasopharynx, where it resides asymptomatically, but it can also spread through the body, causing severe infections (Bogaert *et al.*, 2004). What exactly triggers the pneumococcus to cause infections at these sites in the body is poorly understood. In response to environmental changes, the transcriptional programme is likely to change, which is considered to be niche-specific and can result in expression of distinct virulence factors.

Manganese ions (Mn^{2+}) are important for bacterial life, for instance serving as cofactors for metalloenzymes. The function of these metalloenzymes is widespread among bacterial cellular processes, such as glycolysis, gluconeogenesis and oxidative stress defence (Jakubovics & Jenkinson, 2001). In the pneumococcus, involvement of Mn^{2+} in competence has also been described (Dintilhac *et al.*, 1997), but most studies have focused on its involvement in oxidative stress (Ibrahim *et al.*, 2005; Johnston *et al.*, 2004; McAllister *et al.*, 2004; McCluskey *et al.*, 2004; Paterson *et al.*, 2006; Tseng *et al.*, 2002). In the latter process, Mn^{2+} serves as a cofactor for superoxide

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Abbreviations: CSP, competence-stimulating pepide; PTS, phosphotransferase system; SILAC, stable isotope labelling in cell culture.

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dismutase, an important enzyme that provides defence against superoxide radicals (Archibald & Fridovich, 1981). As the concentration of manganese is much higher in saliva than in blood plasma (Chicharro *et al.*, 1999), fluctuations in the amount of this trace element might serve as a trigger for expression of certain virulence factors.

Various transcriptional regulators have been described for the pneumococcus (Hava *et al.*, 2003b) and several large-scale mutagenesis studies have identified a role in virulence for these regulators (Chastanet *et al.*, 2001; Hava & Camilli, 2002; Hemsley *et al.*, 2003; Lau *et al.*, 2001; Polissi *et al.*, 1998). One of them is encoded by *psaR*, a transcriptional regulator responsive to Mn^{2+} and negatively affecting the expression of the *psa* operon, *pcpA*, *rlrA* and *prtA* (Johnston *et al.*, 2006; Kloosterman *et al.*, 2008). Recently, the PsaR-binding sequence was identified in the promoter region of *psaBCA*, *prtA* and *pcpA*, and a genome-wide screen for this binding sequence did not show any other putative targets (Kloosterman *et al.*, 2008). Moreover, it has been reported that regulation by PsaR is opposite in reaction to two cations, namely repression in high Mn^{2+} and derepression in high Zn^{2+} (Kloosterman *et al.*, 2008).

The *pcpA* gene encodes a choline-binding protein (Sanchez-Beato *et al.*, 1998) and *rlrA* encodes a transcriptional regulator, which controls the expression of the *rlrA* pathogenicity islet (Hava *et al.*, 2003a). The *psaBCA* operon encodes the Psa permease that transports the cations Mn^{2+} and Zn^{2+} into the cell (Dintilhac *et al.*, 1997; Johnston *et al.*, 2004, 2006; Lawrence *et al.*, 1998; McAllister *et al.*, 2004). The permease has been implicated in virulence and protection against pneumococcal infection (Briles *et al.*, 2000; Talkington *et al.*, 1996). Moreover, PsaA has been shown to bind to E-cadherin, a surface molecule of the host cells (Anderton *et al.*, 2007; Romero-Steiner *et al.*, 2003, 2006). PsaR homologues are found in other streptococci, where they regulate homologues of *psaBCA* and contribute to virulence (Hanks *et al.*, 2006; Jakubovics *et al.*, 2000; Paik *et al.*, 2003). The role of PsaR in pneumococcal virulence has been investigated in the strain EF3030 (serotype 19F) genetic background (Johnston *et al.*, 2006). During 7 days of colonization no difference between the wild-type and its isogenic *psaR* mutant was observed, while after 7 days of lung infection, the *psaR* mutant was significantly attenuated compared to the wild-type (Johnston *et al.*, 2006).

In this study, we examined the effect of the deletion of *psaR* on global gene and protein expression in two other pneumococcal strains, D39 (serotype 2) and TIGR4 (serotype 4), using transcriptomics and proteomics. Furthermore, we investigated the contribution of PsaR to pneumococcal virulence in three murine infection models representing the major phases in the life cycle of *S. pneumoniae*: colonization, pneumonia and bacteraemia.

METHODS

Bacterial strains and media. The pneumococcal strains used in this study are listed in Table 1; they were grown at 37 °C in Todd–Hewitt yeast broth (THY), in chemically defined medium (CDM, recipe available on request), or on Colombia base agar (Oxoid) supplemented with 5 % sheep blood (Biotrading). Pneumococcal strains were maintained in 10 % (v/v) glycerol, 10 % skim milk at −80 °C. *Escherichia coli* DH5α (Stratagene) was grown in Luria broth at 37 °C with shaking or on Luria broth agar. Media were supplemented with antibiotics (50 mg ampicillin l^{-1} and/or 20 mg trimethoprim l^{-1}) when appropriate.

Construction of *psaR* mutants. The gene encoding *psaR* was deleted from strain TIGR4 (*sp1638*) and D39 (*spd1450*) by allelic replacement with the *dfr13* cassette conferring trimethoprim resistance (Adrian *et al.*, 2000). To this end, *psaR* with 1000 bp of upstream and downstream flanking sequences was amplified from chromosomal TIGR4 DNA using primer pair psaRSacFw and psaRKnpRv (all primers are listed in Table 2). This amplicon was cloned into pBlueScript KS+ (Stratagene) using the *Sac*II and *Kpn*I restriction sites. The coding sequence of *psaR* was deleted from the plasmid by performing an inverse PCR with primer pair psaRNotFw and psaRSalRv, amplifying the *psaR*-flanking sequences and pBlueScript KS+ and introducing *Not*I and *Sal*I restriction sites for further cloning. This amplicon was ligated to the *dfr13* cassette, which was amplified from pKOT (Hendriksen *et al.*, 2008a) with the primers TmpSalFw and TmpNotRv, to create the knockout construct pKO*psaR*-T4, and transformed into *E. coli* DH5α. A 2620 bp linear DNA fragment containing *psaR*-flanking DNA and *dfr13* was amplified from pKO*psaR*-T4 using primer pair psaRSacFw and psaRKnpRv. This PCR product was used to delete *psaR* from the genome of *S. pneumoniae* TIGR4 by CSP-2-induced (100 ng ml^{-1}) transformation. Transformants were selected on the basis of trimethoprim resistance and were checked by sequencing for recombination at the desired location on the chromosome, i.e. replacement of *psaR* by *dfr3* (which will be transcribed in the opposite direction to *psaR*). Wild-type TIGR4 was subsequently transformed with chromosomal DNA isolated from these Δ*psaR* transformants to rule out the possibility of any additional mutations on the chromosome. The identical procedure was performed for the construction of D39Δ*psaR*, with the exception that the 3′ chromosomal region from TIGR4 of pKO*psaR*-T4 was replaced by the D39-specific 3′ *psaR* region. This region differs between the two

Table 1. Pneumococcal strains used in this study

Strain	Gene identifier	Antibiotic resistance	Reference
D39 wild-type	–	–	NCTC 7466; serotype 2
D39Δ*psaR*	*spd1450*	Trimethoprim	This study
TIGR4 wild-type	–	–	ATCC BAA-334; serotype 4
TIGR4Δ*psaR*	*sp1638*	Trimethoprim	This study

Table 2. Oligonucleotide primers used in this study

Primer name	Sequence (5′–3′)*	Restriction site	Strain
psaRSacFw	GCGCCCGCGGGGAATTTGCATCCTCTTCTCC	*Sac*II	D39/TIGR4
psaRKnpRv	GCGCGGTACCATATTGCCCATCAGCTTTCC	*Kpn*I	TIGR4
psaRNotFw	GCGCGCGGCCGCTCCTCAGTAACGACGAGGATTT	*Not*I	D39/TIGR4
psaRSalRv	GCGCGTCGACGCAGGTCTATGCCAATTTCA	*Sal*I	D39/TIGR4
TmpSalFw	CGCGGTGGTCGACGGATTTTTGTGAGCTTGGACT	*Sal*I	D39/TIGR4
TmpNotRv	GGGGGGCCGCGGCCGCTTACGACGCGCATAGACG	*Not*I	D39/TIGR4
psaRKpnRv-D39	GAAAATGGTACCAGAGAGCAAGAGCCACTC	*Kpn*I	D39
SeqTmpFw	ATAAATGCGGACCGATTCC	–	D39/TIGR4
SeqTmpRv	GCCTTCTTCCCAGTGCTTAAC	–	D39/TIGR4

*Restriction sites on oligonucleotide primers are underlined.

strains: it contains an ISS element in TIGR4, and a small ORF encoding a unique hypothetical protein in D39. Transformation of D39 was induced with CSP-1 (100 ng ml^{-1}).

Transcriptional profiling of D39 and TIGR4 *psaR* mutants. Microarray analysis was performed essentially as described by Hendriksen *et al.* (2007, 2008a). In short, 500 ml CDM was inoculated with 10–20 colonies from agar plates, and these cultures were grown statically at 37 °C. Samples for RNA isolation were taken when the cultures reached an optical density (OD_{600}) of 0.2 (mid-exponential growth). RNA was isolated and purified using the High Pure RNA isolation kit (Roche Diagnostics) as described by Hendriksen *et al.* (2007, 2008a). Contaminating genomic DNA was removed by treatment with RNase-free DNase I (Roche Diagnostics). RNA was isolated from three replicate cultures. Synthesis, subsequent labelling of cDNA, and microarray hybridization were performed as previously described (Hendriksen *et al.*, 2007; Kloosterman *et al.*, 2006). In all cases, dye-swapping was performed with one of the three biological replicates. Microarrays used in this study were constructed as previously described (Hendriksen *et al.*, 2007; Kloosterman *et al.*, 2006) and contain amplicons representing 2087 ORFs of *S. pneumoniae* TIGR4 and 184 ORFs unique for *S. pneumoniae* R6, all spotted in duplicate.

The microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) under GEO Series accession number GSE13505.

DNA microarray data analysis. Dual-channel array images were acquired with a GeneTac LS IV confocal laser scanner (Genomics Solutions) and analysed with ArrayPro 4.5 software (Media Cybernetics). Spots were screened visually to identify those of low quality. Slide data were processed using MicroPreP as previously described (Garcia de la Nava *et al.*, 2003; Hendriksen *et al.*, 2007; van Hijum *et al.*, 2003). Prior to analysis, automatically and manually flagged spots and spots with very low background-subtracted signal intensity [5 % of the weakest spots (sum of Cy3 and Cy5 net signals)] were filtered out of all datasets. Spots with a signal in one channel and no signal in the other were subjected to an empty-value assignment of 1 %, after which net signal intensities were calculated using a grid-based background subtraction. A grid-based Lowess transformation was performed for slide normalization, negative and empty values were removed, and outliers were removed by the deviation test. Further analysis was performed using a Cyber-T Student's *t*-test for paired data (Long *et al.*, 2001). For identification of differentially expressed genes, only genes with a minimum of six reliable measurements, a Bayesian *P*-value <0.001, a false discovery rate (FDR) <0.05, and a standard deviation less than the ratio were included. Since these criteria are purely a statistical measure of differential gene expression and reproducibility across replicates, an additional fold-change cut-off of 2 was applied.

Stable isotope labelling in cell culture (SILAC). For SILAC experiments, D39 wild-type and *psaR*-mutant strains were inoculated in THY and grown to mid-exponential phase. These cultures were used to inoculate CDM, supplemented with both lysine and arginine as the light ($^{12}C_6$ L-lysine, $^{12}C_6$ L-arginine; *psaR* mutant) or heavy ($^{13}C_6$ L-lysine, $^{13}C_6$ L-arginine; wild-type) isotopic counterparts. When these cultures reached an OD_{600} of 0.2, they were diluted to an OD_{600} of 0.04 in fresh pre-warmed CDM with the appropriate heavy or light lysine and arginine, and grown to an OD_{600} of 0.2. This was repeated until the cells had been grown in heavy or light lysine- and arginine-containing CDM for at least five or six generations. After the last generation, the cells were harvested by centrifugation and washed once with PBS. Equal amounts of heavy-labelled wild-type and light-labelled mutant cells were combined and used for mass spectrometry. Bacterial pellets were lysed in lysis buffer [7 M urea, 2 M thiourea, protease inhibitor mix (Roche), pH 8.0]. Lysates were subjected to reduction and alkylation using dithiothreitol and iodoacetamide before LysC and trypsin digestion. Peptide mixtures were purified and desalted using C_{18}-stage tips. Peptide separation and sequence determination was performed with a nano-high-performance liquid chromatography system (Agilent 1100 series) connected to a 7-T linear quadrupole ion trap ion cyclotron resonance Fourier transform mass spectrometer (Thermo Electron). Peptides were separated on a 15 cm 100 μm inner diameter PicoTip emitter for online electrospray (New Objective) packed with 3 μm C18 beads (Reprosil, Dr Maisch GmbH) with a 60 min linear gradient from 2.4 to 40 % acetonitrile in 0.5 % acetic acid at a flow rate of 300 nl min^{-1}. The four most abundant ions were sequentially isolated and fragmented in the linear ion trap by applying collisionally induced dissociation. Proteins were identified using the MASCOT search engine (Matrix Science) against the corresponding *S. pneumoniae* database. MSQuant was used for the quantification and determination of peptide ratios between wild-type and *psaR* mutant. A protein was considered differentially expressed when the results of three technical replicates showed at least a 1.5-fold change in protein abundance and a *P*-value <0.05 (one-sample *t*-test).

Infection models. Nine-week-old female outbred CD-1 mice (Harlan) were used for all infection models. Prior to the infection experiments, D39 and TIGR4 (wild-type and *psaR* mutants) were passaged in mice as described previously (Kerr *et al.*, 2004). Cultures of *S. pneumoniae* D39 and TIGR4 (wild-type and Δ*psaR*) were grown in THY broth to an OD_{600} of 0.3, and stored in aliquots in 10 % glycerol at −80 °C. Prior to infection, these aliquots were spun down and bacteria were resuspended in sterile PBS to 10^6 c.f.u. in volumes

Table 3. Differentially expressed genes/proteins in Δ*psaR* of D39 and/or TIGR4

Ratios are given as $\log_2$-transformed expression of Δ*psaR*/wild-type.

Identifier		Gene	Annotation (http://www.kegg.com)	Microarray		SILAC	
TIGR4	D39			TIGR4	D39	TIGR4	D39
Upregulated							
sp0232	*spd0215*	*infA*	Translation initiation factor IF-1				2.4
sp0303	*spd0277*	*bglA*	6-Phospho-β-glucosidase		1.1		
sp0306	*spd0280*		Transcriptional regulator, putative		1.2		
sp0308	*spd0281*		PTS system, cellobiose-specific IIA component		1.5		
sp0310	*spd0283*		PTS system, cellobiose-specific IIC component		1.2		
–	*spd0322*	*cps2G*	Glycosyltransferase, group 1 family protein				4.5
sp0459	*spd0420*	*pflB*	Formate acetyltransferase			0.8	
sp0461	–	*rlrA*	Transcriptional regulator	2.7			
sp0462	–	*rrgA*	Cell wall surface anchor family protein	3.1			
sp0463	–	*rrgB*	Cell wall surface anchor family protein	3.7		2.0	
sp0464	–	*rrgC*	Cell wall surface anchor family protein	3.7			
sp0467	–		Sortase SrtC, putative	2.5			
sp0468	–		Sortase SrtD, putative	2.6			
sp0641	*spd0558*	*prtA*	Serine protease, subtilase family PrtA	3.6	3.0		3.9
sp0869	*spd0764*	*sufS*	Cysteine desulfurases, SufS subfamily protein				5.0
sp1244	*spd1101*	*ftsY*	Signal recognition particle-docking protein FtsY				7.6
sp1517	*spd1345*	*greA*	Transcription elongation factor GreA				2.9
sp1531	*spd1360*		Hypothetical protein				2.7
sp1636	*spd1448*		Rrf2 family protein	1.3			
sp1637	*spd1449*		Hypothetical protein	1.4	1.2		
sp1647	*spd1460*	*pepO*	Endopeptidase O			0.6	
sp1648	*spd1461*	*psaB*	Manganese ABC transporter, ATP-binding protein	3.6	3.3		
sp1649	*spd1462*	*psaC*	Manganese ABC-transporter permease	2.8	2.4		
sp1650	*spd1463*	*psaA*	ABC transporter, substrate-binding lipoprotein			5.4	5.1
sp1805	*spd1591*		Hypothetical protein			0.6	
sp1856	*spd1637*		Transcriptional regulator, MerR family	1.2			
sp1857	*spd1638*	*czcD*	Cation-efflux system protein	1.7			
sp2136	*spd1965*	*pcpA*	Choline-binding protein PcpA	4.0	4.6	7.4	
sp2210	*spd2037*	*cysK*	Cysteine synthase A				4.0
Downregulated							
sp0112	*spd0109*		Polar amino acid transport system substrate-binding protein		−1.2		
sp0138	*spd0141*		Hypothetical protein		−1.7		
sp0139	*spd0142*		Hypothetical protein		−1.8		
sp0140	*spd0143*	*ugd*	UDP-glucose/GDP-mannose dehydrogenase		−1.7		
sp0141	*spd0144*	*mutR*	Positive transcriptional regulator of MutA		−1.2		
sp0142	*spd0145*		Hypothetical protein		−1.0		
sp0143	*spd0146*		Hypothetical protein		−1.9		
sp0144	*spd0147*		Hypothetical protein		−1.5		
sp0145	*spd0148*		Hypothetical protein		−2.0		
sp0146	*spd0149*		Hypothetical protein		−1.7		
sp0148	*spd0150*		ABC transporter, substrate-binding protein			−2.1	
sp0524	*spd0466*		BlpT protein, fusion		−1.5		
sp0525	*spd0467*	*blpS*	BlpS protein		−1.4		
sp0526	*spd0468*	*blpR*	Response regulator BlpR (TCS13)		−1.2		
sp0527	*spd0469*	*blpH*	Sensor histidine kinase BlpH, putative (TCS13)		−1.1		
sp0529	*spd0471*	*blpB*	BlpC ABC transporter		−1.5		
sp0530	*spd0472*	*blpA*	BlpA, pseudogene		−1.5		
sp0533	*spd0046*	*blpK*	Bacteriocin BlpU (highly similar to *sp0533*)		−1.5		
sp0541	*spd0046*	*blpO*	Bacteriocin BlpO (5′ highly similar to *sp0541*)		−1.5		
sp0545	*spd0473*	*blpY*	Immunity protein BlpY		−2.4		
sp0546	*spd0474*	*blpZ*	BlpZ protein, fusion		−1.2		

Table 3. cont.

Identifier		Gene	Annotation (http://www.kegg.com)	Microarray		SILAC	
TIGR4	D39			TIGR4	D39	TIGR4	D39
sp0547	*spd0475*		Hypothetical protein		−2.4		
sp0664	*spd0577*	*zmpB*	Zinc metalloprotease ZmpB, putative			−0.8	
sp0798	*spd0701*	*ciaR*	DNA-binding response regulator CiaR		−1.1		
sp0925	*spd0817*		Hypothetical protein		−1.4		
sp1024	*spd0910*	*glyA*	Serine hydroxymethyltransferase		−1.1		
sp1249	*spd1107*		GMP reductase	−1.0			
sp1458	*spd1287*	*trxB*	Thioredoxin reductase			−1.0	
sp1543	*spd1372*		Glyoxalase family protein		−1.5		
sp1638	*spd1450*	*psaR*	Iron-dependent transcriptional regulator, PsaR	−2.7	−2.0		
sp1802	*spd1588*		Hypothetical protein		−1.2		
sp1804	*spd1590*		General stress protein 24, putative		−1.3		
sp1923	*spd1726*	*ply*	Pneumolysin	−1.0			
sp2022	*spd1831*		PTS system, cellobiose-specific IIC component, putative	−1.4			
sp2023	*spd1832*		PTS system, cellobiose-specific IIB component, putative	−1.2			

depending on the infection model used. Upon intranasal infection, mice were anaesthetized with 2.5 % (v/v) isoflurane/O_2. At predetermined time points after infection, groups of mice were sacrificed by cervical dislocation and samples of various sites were taken to determine the bacterial load. During infection, signs of disease were closely monitored. If animals reached a moribund state, they were sacrificed by cervical dislocation and excluded from the experiment prematurely. All animal experiments were performed with approval from the Animal Experimentation Committee (DEC) of Erasmus Medical Centre, Rotterdam, The Netherlands.

Colonization model of infection. In the colonization model, 10 µl PBS containing 10^6 c.f.u. bacteria was administered to the nostrils of groups of five mice as described previously (Hendriksen *et al.*, 2008b; Kadioglu *et al.*, 2000). Due to this small volume, only the nose of the mice becomes infected. Bacteria were recovered from the nasopharynx by flushing the nose with 2 ml sterile PBS (Kerr *et al.*, 2004), and lungs were removed from the body and homogenized in 2 ml sterile PBS using a hand-held homogenizer (Polytron PT 1200, Kinematica AG). Viable bacteria from the nasal lavage fluid and homogenized lung samples were counted by plating serial 10-fold dilutions on Colombia blood agar plates. Time points for sampling were 30 min, 24 h, 48 h, 96 h and 192 h post-infection. The 30 min time point is considered to be the start of the infection, and is therefore referred to as $t=0$. Bacteriology results are expressed as geometric mean ± SEM. Comparison of bacterial loads in the time-course experiment was performed using a Student's *t*-test with $P<0.05$ considered statistically significant.

Pneumonia model of infection. In the pneumonia model, five mice per group were infected with 50 µl PBS containing 10^6 c.f.u. pneumococci as described previously (Hendriksen *et al.*, 2008b). Bacteria were recovered from the different sites as described above, with the addition of a blood sample obtained by cardiac puncture. Time points for sampling were 0, 12, 24 and 36 h post-infection. Viable bacteria isolated from the nasal lavage fluid, homogenized lungs and blood were quantified as described above. Bacteriology results are expressed as geometric mean ± SEM. Comparison of bacterial loads in the time-course experiment was performed using a Student's *t*-test with $P<0.05$ considered statistically significant.

Bacteraemia model of infection. In the bacteraemia model, groups of ten mice were infected in a tail vein with 10^6 c.f.u. resuspended in 100 µl sterile PBS as described previously (Hendriksen *et al.*, 2008b). Bacteria were recovered from the blood by a lateral tail vein puncture from the same mouse at three predetermined time points after infection (0, 12, 24 h) and by a cardiac puncture at the last time point, 36 h. In addition, mouse survival times were scored, after which analysis of survival times was performed using the log-rank test with $P<0.05$ considered statistically significant.

RESULTS

Transcriptional analyses of Δ*psaR* in D39 and TIGR4

By means of microarrays analysis, we assessed which genes were affected in expression due to the mutation of *psaR* in two genetic backgrounds, i.e. D39 and TIGR4. To this end, transcriptional profiles of wild-type strains were compared to their isogenic Δ*psaR* strains. These bacteria were grown in chemically defined medium (CDM) and harvested at mid-exponential growth phase. In all experiments, the *psaR* mutant strains grew like the wild-type. The concentration of Mn^{2+} in CDM is 180 µM, which is sufficient for PsaR regulation (see Kerr *et al.*, 2004, where 50 µM was used). For comprehensibility, loci of D39 are referred to by the TIGR4 gene identifiers (in Table 3 both annotations are given). Comparison of transcriptional profiles of D39 and TIGR4 wild-type with their Δ*psaR* counterparts revealed 19 differentially expressed genes in TIGR4Δ*psaR*, and 37 in D39Δ*psaR*. Of these, five genes were upregulated in both TIGR4 and D39 *psaR* mutants, while only *psaR* itself was downregulated in both strains (Fig. 1).

The genes that were differentially expressed in both serotypes were all upregulated in the *psaR* mutant, confirming the general role of PsaR as a transcriptional repressor (Johnston *et al.*, 2006; Kloosterman *et al.*, 2008). Among those were the previously described targets, the *psa* operon (*sp1648–sp1650*), *pcpA* (*sp2136*) and *prtA* (*sp0614*).

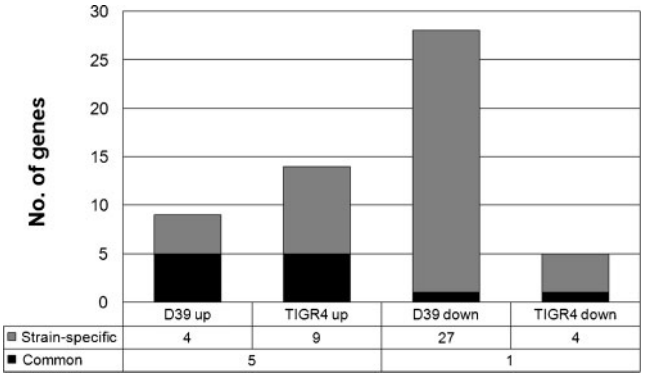

Fig. 1. Number of genes differentially expressed in D39Δ*psaR* and TIGR4Δ*psaR*.

In addition, *sp1637*, encoding a hypothetical protein of unknown function, was found to be upregulated in the *psaR* mutants of both strains (Table 3).

Four genes were found to be repressed by PsaR in D39 (i.e. upregulated in D39Δ*psaR*) only: *sp0303*, encoding 6-phospho-*β*-glucosidase; *sp0306*, encoding a putative transcriptional regulator; and two genes of a putative operon encoding a cellulose-specific phosphotransferase system (PTS) (*sp0308* and *sp0310*).

Twenty-seven genes were downregulated in D39Δ*psaR*. This set of genes contained *sp0112*, predicted to encode an amino acid substrate-binding protein; the transcriptional regulator *mutR* (*sp0141*); a putative bacteriocin system (*sp0142–sp0146*); the *blp* two-component system (TCS13; *sp0526–sp0527*); the gene encoding response regulator CiaR (*sp0798*); and *glyA* (*sp1024*) encoding serine hydroxymethyltransferase. The full set of differentially expressed genes in D39 is listed in Table 3.

In addition to the common gene targets, nine genes were specifically upregulated in the *psaR* mutant in TIGR4 (Fig. 1). These included the *rlrA* pathogenicity islet (*sp0461–sp0468*) as reported previously (Johnston *et al.*, 2006); *sp1636* (encoding an Rf2 family protein); and two adjacent genes, encoding a MerR family transcriptional regulator (*sp1856*) and *czcD* (*sp1857*), a Zn^{2+}-efflux pump (Table 3).

Four genes were downregulated: *guaC*, encoding GMP reductase (*sp1249*); *ply*, encoding pneumolysin (*sp1923*); and two genes encoding the B and C components of a putative cellobiose-specific PTS (*sp2022* and *sp2023*). The complete set of differentially expressed genes in TIGR4Δ*psaR* is given in Table 3.

Proteome analysis of Δ*psaR* in D39 and TIGR4

To examine if the observed PsaR-mediated differences in gene expression corresponded to changes in protein expression, we performed SILAC for both wild-type strains and their *psaR* mutants. To this end, wild-type and isogenic Δ*psaR* counterparts were cultured in the presence of stable isotope-labelled (heavy, $^{13}C_6$) or normal (light, $^{12}C_6$) L-lysine and L-arginine, respectively. Equal amounts of bacteria were mixed and analysed by mass spectrometry, after which changes in protein expression between wild-type and isogenic Δ*psaR* strains were derived from the corresponding heavy to light peptide ratios.

In D39Δ*psaR*, nine proteins were found to be more abundant than in the wild-type (Table 3). Most pronounced were FtsY, PsaA, SufS, Cps2G, CysK and PrtA, all of which displayed expression levels at least eight times higher in the *psaR* mutant than in the wild-type. No proteins were identified that were less abundant in D39Δ*psaR* (Table 3).

In TIGR4Δ*psaR*, six proteins were more abundant than in the wild-type. Most pronounced were PcpA, PsaA and RrgB, all with at least a fourfold increase in expression in the *psaR* mutant (Table 3). Three proteins were less abundant in TIGR4Δ*psaR*, i.e. an ABC transporter of a putative bacteriocin system (*sp0148*), TrxB and ZmpB (Table 3).

PsaR does not contribute to pneumococcal colonization

To assess the contribution of PsaR regulation to pneumococcal virulence, we examined the phenotypes of the two wild-type strains and their *psaR* mutants in three murine models of infection. In the colonization model, both wild-type D39 and TIGR4 were capable of extended colonization of the murine nasopharynx for a period of 192 h. The level of colonization was fairly consistent during this period, varying between 10^4 and 10^6 c.f.u. ml^{-1} (Fig. 2).

We did not observe a clear effect of the deletion of *psaR* on the colonization ability of strains: comparable bacterial loads of wild-type and mutant strains were found during 192 h of colonization, with bacterial loads between 2.5×10^4 c.f.u. ml^{-1} and 3.2×10^5 c.f.u. ml^{-1} for D39 wild-type and its isogenic *psaR* mutant, and between 1.3×10^5 c.f.u. ml^{-1} and 5.0×10^5 c.f.u. ml^{-1} for TIGR4 wild-type and its Δ*psaR* derivative (Fig. 2a and b). The only exception was a small but significantly higher load ($P=0.0049$) of D39Δ*psaR* at 24 h (Fig. 2b).

PsaR is not required for pneumococcal pneumonia

In the pneumonia model, we observed a similar trend in bacterial loads of the nasopharynx as was observed in the colonization model, although the actual numbers of bacteria were lower. At 12 h of infection there was a small but significantly higher nasopharyngeal load in mice infected with D39Δ*psaR* as compared to wild-type (data not shown). For TIGR4 wild-type and Δ*psaR*, small but significant differences in bacterial load were observed throughout the entire experiment (data not shown).

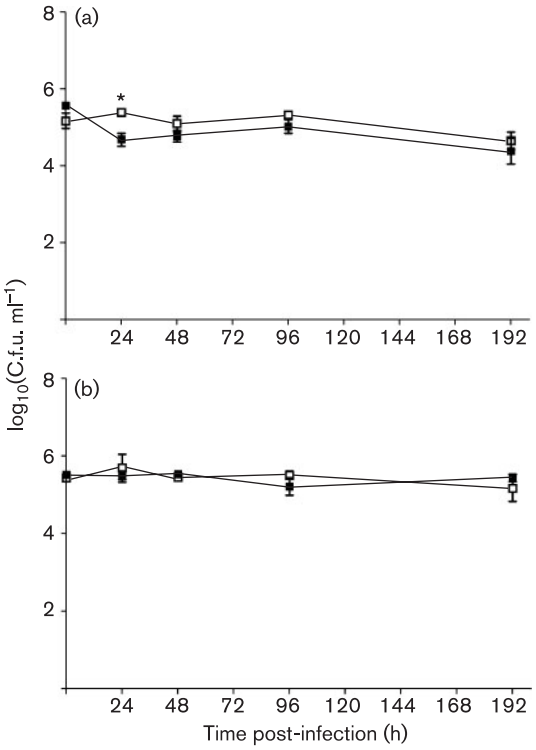

Fig. 2. Colonization model. Bacterial loads in the nasal lavage fluid of mice infected with (a) D39 wild-type (■) and D39Δ*psaR* (□), (b) TIGR4 wild-type (■) and TIGR4Δ*psaR* (□). *, $P<0.05$.

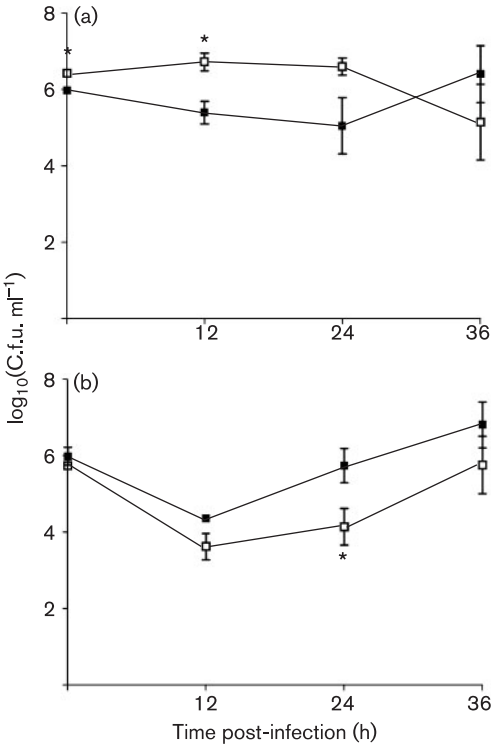

Fig. 3. Pneumonia model. Bacterial loads in lungs and blood of mice infected with (a) D39 wild-type (■) and D39Δ*psaR* (□), (b) TIGR4 wild-type (■) and TIGR4Δ*psaR* (□). *, $P<0.05$.

Furthermore, bacterial loads in the nasopharynx were comparable to those of the first 36 h in the colonization model (data not shown).

We did not observe any clear difference in the bacterial survival in the lungs between wild-type and *psaR* mutants (Fig. 3a). However, immediately after infection (0 h) and 12 h post-infection, the lung homogenates of D39Δ*psaR*-infected mice had significantly higher bacterial loads than those of wild-type infected mice: 1.2×10^5 vs 6.3×10^4 c.f.u. ml^{-1} at 0 h, and 1.5×10^5 vs 5.8×10^4 c.f.u. ml^{-1} at 12 h. This suggests that there might be an initial positive effect of the *psaR* mutation, as the inoculum of the wild-type contained (more than) twice as many bacteria as that of the *psaR* mutant (1.2×10^6 and 4.5×10^5 c.f.u. ml^{-1}, respectively).

In contrast, mice infected with the TIGR4 *psaR* mutant had lower bacterial loads in the lungs compared to mice infected with TIGR4 wild-type, although these differences were only statistically significant at 24 h post-infection ($P=0.0401$) (Fig. 3b).

The number of bacteria that were able to reach the systemic circulation was not significantly different between wild-type and Δ*psaR* in either D39 or TIGR4 (data not shown), indicating that PsaR regulation is not required for the transition from the lungs to the systemic circulation.

PsaR contributes to survival of pneumococci during bacteraemia

The most prominent phenotype of the *psaR* mutants was observed in the bacteraemia model of infection. D39 wild-type-infected mice had significantly more bacteria in the blood than the D39Δ*psaR*-infected mice during the first 24 h of infection (Fig. 4a), while variation in the inocula was marginal (1.6×10^6 for wild-type and 1.4×10^6 for *psaR* mutant). In contrast, no differences were seen between mice infected with TIGR4 wild-type and TIGR4Δ*psaR* during the first 24 h of infection. At 36 h post-infection, however, TIGR4 wild-type-infected mice had twice as many bacteria in the bloodstream ($P=0.0396$) (Fig. 4b).

In addition to the differences in bacterial load, we also observed a difference in murine survival after infection with D39 or TIGR4 in our bacteraemia model: all TIGR4-infected mice (wild-type and Δ*psaR*) survived, in contrast to D39 wild-type infected mice, which died within 30 h. Of the D39Δ*psaR*-infected mice, three survived the experiment. Consequently, the median survival time of D39Δ*psaR*-infected mice was significantly longer than that of mice infected with its wild-type parental strain (the median survival for Δ*psaR* was 24 h and for wild-type 30 h, with $P=0.0405$ and hazard ratio=1.866).

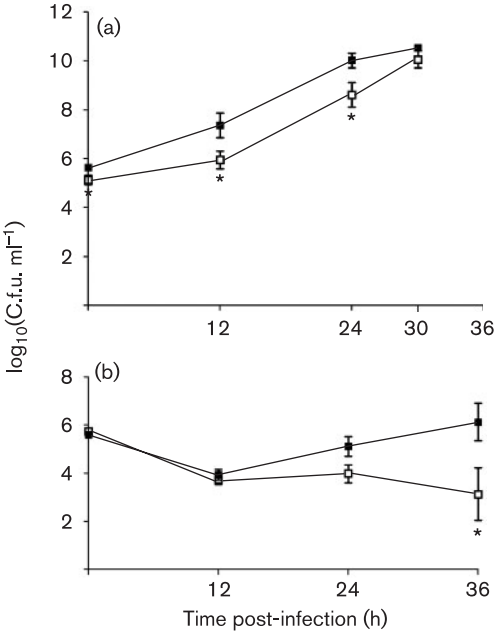

Fig. 4. Bacteraemia model. Bacterial loads in blood of mice infected with (a) D39 wild-type (■) and D39Δ*psaR* (□), (b) TIGR4 wild-type (■) and TIGR4Δ*psaR* (□). *, $P<0.05$.

DISCUSSION

During colonization and infection of the human host, *S. pneumoniae* encounters fluctuating amounts of free Mn^{2+} in the different niches where the bacterium resides. Since Mn^{2+} is required for several cellular processes, proper regulation of manganese homeostasis is vital for pneumococcal physiology and virulence. The transcriptional repressor PsaR has been described to play an important role in this process, at least in the genetic background of strain EF3030 (serotype 19F) (Johnston *et al.*, 2006). However, we and others have previously shown that, even though transcriptional regulators themselves appear to be conserved between *S. pneumoniae* strains, they often have a strain-specific impact on global transcription and virulence (Hendriksen *et al.*, 2007; Blue & Mitchell, 2003; McCluskey *et al.*, 2004). To examine whether a similar strain specificity holds true for PsaR, we identified its targets and its contribution to experimental virulence in two additional strains, TIGR4 and D39.

We used a combination of transcriptional and proteome analyses for the identification of PsaR targets. Observed discrepancies between these two approaches could be the result of several factors, such as low levels of gene expression, protein instability, or post-translational regulation. The previously reported PsaR targets, the Psa operon, *pcpA* and *prtA* (Johnston *et al.*, 2006; Kloosterman *et al.*, 2008), were confirmed in TIGR4 and D39 by both transcriptomics and proteomics. In addition, we found *sp1637*, encoding a hypothetical protein, to be upregulated in both *psaR* mutants. Since the latter gene is located directly upstream of *psaR* (*sp1638*), we cannot entirely exclude the possibility that the derepression of *sp1637* is caused by transcriptional read-through of the trimethoprim-resistance cassette used to create the *psaR* knockout. We have previously shown that the common PsaR target *pcpA* is required for adherence to the human Detroit epithelial cell line and that the expression of this gene is also directly positively regulated by the nutritional regulator CodY (Hendriksen *et al.*, 2008a). The inability of the *codY* mutant to colonize the murine nasopharynx underscored, albeit indirectly, the involvement of PcpA in adherence and colonization. However, a recent study showed that PcpA is not involved in colonization but has a role in invasive disease (Glover *et al.*, 2008). These contradictory results are possibly due to different experimental set-ups; however, the exact role of PcpA during pneumococcal pathogenesis remains unclear.

In addition to the shared PsaR targets (i.e. the overlapping genes between D39 and TIGR4), several genes were differentially expressed in D39 or TIGR4 only. This strain-specific differential expression might be due to direct regulation by PsaR, but is more likely to be either an indirect effect caused by an imbalance in Mn^{2+}/Zn^{2+} homeostasis due to the lack of PsaR or, possibly, downstream signalling of other regulators. Moreover, it seems that this effect was more severe for D39, since more genes and proteins were affected in their expression level in this strain. Downstream regulation or a disturbed balance in other cellular processes might be the cause of this differential expression, since manganese cations have been shown to be required for several bacterial cellular processes (Jakubovics & Jenkinson, 2001). For example, manganese cations have been shown to function as cofactors for enzymes in glycolysis, amongst others 6-phospho-β-glucosidase (Varrot *et al.*, 1999). The genes encoding 6-phospho-β-glucosidase and a PTS downstream of it (*sp0303*, *sp0306* and *sp0308*), were upregulated in D39Δ*psaR*, in line with indirect regulation. However, in D39 these genes are strongly downregulated in the presence of Zn^{2+}, which is not in agreement with the opposite effect of Mn^{2+} and Zn^{2+} on PsaR regulation (Kloosterman *et al.*, 2008).

In D39Δ*psaR*, the gene *ciaR*, involved in competence development, was upregulated. It has been reported that Mn^{2+} is required for genetic transformation (Dintilhac *et al.*, 1997), suggesting that downregulation of this gene might be an indirect effect of the *psaR* mutation. Several other systems were downregulated in D39Δ*psaR* alone, such as a putative bacteriocin system (*sp0142–sp0146*), the *blp* bacteriocin system, and the *blp* two-component system (TCS13). The genes *sp0142–sp0146* have also been shown to be regulated by the nutritional regulator CodY in D39 (Hendriksen *et al.*, 2008a). Possibly, the downregulation of the *blp* bacteriocin genes is due to downregulation of the *blp* two-component system (Dawid *et al.*, 2007; de Saizieu *et al.*, 2000).

Notably in TIGR4, a MerR family regulator (*sp1856*) and *czcD* (*sp1857*), encoding a Zn^{2+}-efflux system, were upregulated in the *psaR* mutant. These two genes have also been shown to be upregulated in the presence of Zn^{2+}, underscoring the reported opposite effect of Mn^{2+} and Zn^{2+} on PsaR regulation (Kloosterman *et al.*, 2008). The *czcD* gene has recently been shown to be regulated by SczA (*sp1858*) in reaction to increasing cellular zinc concentrations (Kloosterman *et al.*, 2007), but this efflux system might also be involved in Mn^{2+} homeostasis. This again indicates that these regulatory systems of cation homeostasis are intertwined (Kloosterman *et al.*, 2008).

The expression of *ply* (encoding the pneumococcal toxin pneumolysin) was downregulated as a result of the *psaR* mutation during *in vitro* growth in TIGR4 only. If this downregulation also occurred during our infection experiments, it did not have a large effect on experimental virulence of the TIGR4 strain, as the Δ*psaR* mutant was as virulent as the wild-type in all our infection models. Only at 24 h post-infection during experimental pneumonia did mice infected with the *psaR* mutant have significantly lower bacterial loads in the lungs compared to those infected with the wild-type.

A role for PsaR in virulence has been reported in a strain EF3030 genetic background (Johnston *et al.*, 2006). During 7 days of colonization no difference between the wild-type and isogenic *psaR* mutant was observed, which is in agreement with our results. Johnston *et al.* (2006) reported that during lung infection the *psaR* mutant had significantly lower bacterial loads than the wild-type after 7 days of infection. However, in our pneumonia model, we observed higher bacterial loads in D39Δ*psaR*-infected mice at the beginning of the experiment (the first 12 h). The TIGR4 Δ*psaR*-infected mice had significantly lower bacterial loads at 24 h only. Taking the results together, we did not observe any clear role for PsaR during lung infection in our study.

Upon intravenous infection, we observed the most pronounced effect of deletion of *psaR*. The *psaR* mutant was attenuated at the early stages of blood infection. In line with this, PsaR was identified by a large signature-tagged mutagenesis study to be required for full virulence of a serotype 3 strain in a bacteraemia model of infection after 24 h infection (Lau *et al.*, 2001). Importantly, for D39 no significant difference was observed in our model at 30 h post-infection. This indicates that in the first 24 h PsaR regulation is required for adaptation to the blood. Furthermore, in D39, survival times of wild-type and Δ*psaR*-infected mice were different, although this just reached statistical significance. In contrast, mice infected with TIGR4 wild-type and Δ*psaR* did not exhibit any differences in the first 24 h of experimental bacteraemia, while after 36 h they did. This indicates that in TIGR4, PsaR contributes to the later stages of bacteraemia. However, since the TIGR4 wild-type was not able to cause infection as severe as D39, this contribution is considered to be marginal.

Although we observed disturbed gene regulation in the D39 and TIGR4 mutants, in most infection models (described above) we did not observe large effects on virulence. A study performed on the pneumococcal carbohydrate regulator RegM showed that, even though deletion of a regulator does not necessarily lead to large differences in expression of known virulence genes, it can affect pneumococcal virulence (Giammarinaro & Paton, 2002). Conversely, a second study by Trombe and co-workers showed that deleting the global regulator RegR does not dramatically affect virulence, but has a large effect on gene expression (Chapuy-Regaud *et al.*, 2003). It seems that the pneumococcus has several compensatory virulence pathways.

Strain-specific contributions of genes encoding transcriptional regulators have been reported previously and complicate ascribing a general role for these genes in transcriptional control of their targets and their contribution to virulence (Hendriksen *et al.*, 2007; McCluskey *et al.*, 2004). PsaR directly controls the expression of a select group of genes individually important for virulence (*psaBCA* and *rlrA*) (Johnston *et al.*, 2006; Kloosterman *et al.*, 2008). The observed differences in PsaR-regulated gene expression between D39 and TIGR4 might be the indirect effect of an unbalanced Mn^{2+} homeostasis, which suggests that physiological differences might reflect genetic differences present in these strains. A clear example of the genetic difference is the *rlrA* pathogenicity islet, which is strongly upregulated in TIGR4Δ*psaR*. However, the genetic diversity between strains only varies as much as 10 %, most of which can be attributed to the capsular genes (Hakenbeck *et al.*, 2001; Lanie *et al.*, 2007). In conclusion, PsaR does not contribute to colonization of *S. pneumoniae*, but it is involved in invasive disease where it has a strain-specific impact during both pneumonia and bacteraemia.

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